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N-ethylmaleimide sensitive factor is required for fusion of the *C. elegans* uterine anchor cell

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Abstract

The fusion of the *Caenorhabditis elegans* uterine anchor cell (AC) with the uterine-seam cell (utse) is an excellent model system for studying cell–cell fusion, which is essential to animal development. We obtained an egg-laying defective (Egl) mutant in which the AC fails to fuse with the utse. This defect is highly specific: other aspects of utse development and other cell fusions appear to occur normally. We find that defect is due to a missense mutation in the *nsf-1* gene, which encodes *N*-ethylmaleimide-sensitive factor (NSF), an intracellular membrane fusion factor. There are two NSF-1 isoforms, which are expressed in distinct tissues through two separate promoters. NSF-1L is expressed in the uterus, including the AC. We find that *nsf-1* is required cell-autonomously in the AC for its fusion with the utse. Our results establish AC fusion as a paradigm for studying cell fusion at single cell resolution and demonstrate that the NSF ATPase is a key player in this process.

Keywords: NSF; AC; utse; Cell fusion; *C. elegans*

Introduction

The enclosure of cells' contents by phospholipid membranes is central to life, and eukaryotic cells also contain internal organelles that are bounded by membranes. Intracellular vesicular transport establishes these phospholipid boundaries and requires the fission and fusion of membranes. The well-known proteins required for vesicular fusion include NSF (*N*-ethylmaleimide-sensitive factor), SNAPs (soluble NSF attachment proteins), and SNARE (SNAP receptor) complexes. SNAREs are membrane proteins that are specific to either the vesicular or target membrane and assemble with one another during membrane fusion (Bennett, 1995; Clary et al., 1990; Malhotra et al., 1988). NSF is broadly required for intracellular membrane fusion and functions by binding to SNARE complexes through SNAPs (Clary et al., 1990; reviewed in Whiteheart and Matveeva, 2004). This leads to the disassembly of the SNARE complexes, permitting the reutilization of individual SNAREs (Sollner et al., 1993).

Although many different SNAPs and SNAREs have been identified, some with tissue-specific expression, each organism contains only one or two *NSF* genes. In general, NSF appears to be particularly abundant in the nervous system. *Drosophila* contains two paralogous *NSF* genes, which have some redundant functions. In addition, one of these is specific to the nervous system at the adult stage (*dNSF1*), while the other functions in the mesoderm in larvae (*dNSF2*), one of whose fates is syncytial muscle (Golby et al., 2001; Pallanck et al., 1995). In addition, mouse seems to make two *NSF* transcripts from a single *NSF* gene, probably through alternative splicing (Puschel et al., 1994). The transcripts are largely found in the nervous system.

Recently, NSF has also been reported to associate with non-SNARE receptors and influence their transport. Examples include the GluR2 subunits of the AMPA receptors (AMPARs) (Hanley et al., 2002; Huang et al., 2005; Lee et al., 2002) and β2-adrenergic receptors whose recycling might also involve another interaction of NSF with β-arrestins 1 and 2 (Cong et al., 2001; reviewed in Shenoy and Lefkowitz, 2003). In addition,

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NSF also binds a number of intracellular constituents including small GTP-binding proteins of the Rab family. In at least some cases described above, NSF may promote disassembly of complexes, such as AMPAR-PICK1 (protein interacting with C-kinase-1) (Hanley et al., 2002) and γ-SNAP-Gaf-1/Rip11 (Tani et al., 2003; reviewed in Whiteheart and Matveeva, 2004), similar to its role with SNAREs.

Just as transporting vesicles fuse, the plasma membranes that separate cells can also fuse (Fig. 1A). Cell–cell fusion is critical to animal development, including sperm–egg fusion during fertilization and cell–cell fusion during formation of placenta, muscles, and bones (reviewed in Shemer and Podbilewicz, 2000). In addition, it has been shown that stem cell and tumor cell fusion is important for stem cell transdifferentiation and cancer progression, respectively (Camargo et al., 2003; reviewed in Chen and Olson, 2005; reviewed in Duelli and Lazebnik, 2003). Intensive research has revealed several fusogenic proteins used by enveloped viruses for entry into host cells as well as their mechanisms of function (reviewed in Hernandez et al., 1996). Much less is known about the molecular mechanisms of cell–cell fusion in multicellular organisms. In general, viral fusogenic proteins each contain a hydrophobic stretch that can associate with target membranes. However, there are no consensus sequences shared among virus families (reviewed in Hernandez et al., 1996).

To date, several candidates for cell–cell fusion proteins in multicellular organisms, such as *Caenorhabditis elegans*, *Drosophila*, mouse, and human, have been proposed (reviewed in Potgens et al., 2002; Schultz and Williams, 2005 and Stein et al., 2004). Izumo (Inoue et al., 2005), tetraspanin protein family members, such as CD9 (Hemler, 2003), and ADAM (a disintegrin and metalloprotease) protein family members including fertilins and cyritestin have been implicated in sperm–egg fusion. Izumo and CD9 have been shown to be necessary for the fusion event, while ADAMs are suggested to be involved in sperm–egg binding rather than in their fusion. Several immunoglobulin superfamily members, such as Duf, Rst, Sns, and Hbs, have been proposed to be essential adhesion molecules for myoblast fusion, an essential aspect of muscle formation (reviewed in Chen and Olson, 2004, 2005; Taylor, 2000). In addition, human endogenous retrovirus envelope proteins, Syncytins have been shown to be fusogenic and involved in trophoblast fusion (Blaise et al., 2003; Dupressoir et al., 2005; Mi et al., 2000; reviewed in Potgens et al., 2002). All of the above observations strongly imply that a diversity of proteins are involved in cell–cell fusion and suggest that the full range of fusogenic proteins and/or fusion mechanisms may not yet be known.

The *eff-1* gene in *C. elegans* has recently been found to be required for epithelial cell fusion (Mohler et al., 2002). EFF-1 is an integral membrane protein that, like viral fusion proteins, contains a short hydrophobic sequence (Mohler et al., 2002). Ectopic expression of EFF-1 causes cells that would not normally fuse to do so (del Campo et al., 2005; Shemer et al., 2004), suggesting that this protein is sufficient to induce cell fusion. While *eff-1* is required broadly for *C. elegans* cell fusions, it appears not to function in every fusion-fated tissue. For instance, sperm–egg fusion does not appear to require EFF-1 (del Campo et al., 2005). In addition, *eff-1* mutants do not show defects in the process of AC/utse fusion discussed below.

The reproductive system of the *C. elegans* hermaphrodite is comprised of a number of tissues whose development is coordinated through cell–cell interaction. The uterine anchor cell (AC) plays a central role in the development of this system. The AC/VU (Ventral Uterine precursor cell) decision, which is completed through LIN-12/Notch and LAG-2/Delta lateral signaling, ensures that only a single cell in each hermaphrodite uterus becomes an AC (Greenwald et al., 1983; Kimble, 1981; Seydoux and Greenwald, 1989; Wilkinson et al., 1994). Subsequently, the AC initiates vulval development by inducing

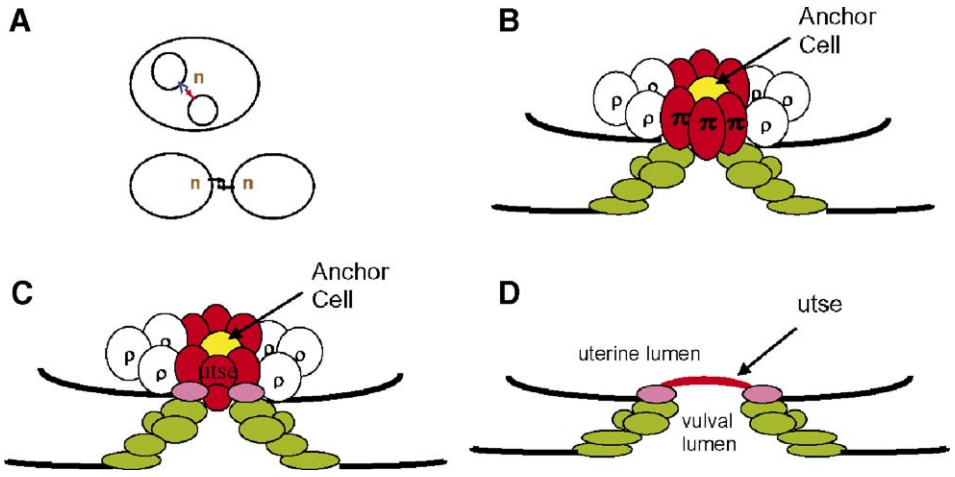

Fig. 1. Membrane fusion and uterine–vulval development. (A) Intra- (upper) vs. inter- (lower) cellular membrane fusion. The ovals and the circles indicate cells and vesicles, respectively. red t: t-SNARE, blue v: v-SNARE, brown n: NSF, green f: cell fusogens. Cell fusogens functioning in AC/utse fusion are currently unknown. Following vesicle-to-plasma membrane or vesicle-to-vesicle contact, the cytoplasmic localization of NSF enables it to disassemble SNAREs. In cell–cell fusion, NSF would need to be outside the cell in order to disassemble fusogens. (B–D) uterine–vulval development during the L3 to L4 stage. Modified from Newman et al. (2000). (B) The AC induces the adjacent six VU granddaughters to adopt π cell fates (red) and others become ρ cells (white). (C, D) π cells divide once to produce 12 π cell daughters. Eight of them (red) fuse together to form the utse to which the AC fuses and the rest become uv1 cells (pink).

three of six vulva precursor cells (VPCs) to adopt vulval fates via the epidermal growth factor (EGF)-like LIN-3 (Hill and Sternberg, 1992; Kimble, 1981; Sternberg and Horvitz, 1986). In addition, the AC induces 6 of 12 VU granddaughters to adopt π cell fates using unidirectional LIN-12/Notch and SEL-12 Presenilin signaling (Cinar et al., 2001; Newman et al., 1995; reviewed in Newman and Sternberg, 1996). After π cells divide once to give 12 π cell daughters, eight of these daughters fuse together to form the syncytial uterine-seam cell (utse) (Fig. 1) (Newman et al., 1996). The thin laminar process of the utse separates the uterine and vulval lumens and is broken by the first egg that is laid (Newman et al., 1996). The proper differentiation of the utse is thus necessary for egg laying. A final critical step towards establishing a proper uterine–vulval connection is fusion of the AC with the utse. If this fails to occur, the AC blocks access between the two tissues (Newman et al., 1996).

The fusion of the AC with eight π cell progeny is an excellent model system for studying cell–cell fusion since the lineage of *C. elegans* is generally invariant (Kimble, 1981; Sulston and Horvitz, 1977; Sulston et al., 1983), development can be observed at single cell resolution, and the AC is easy to find. Further, the requirement of a functional uterine–vulval connection for egg laying provides a powerful basis for genetic screens. A number of mutants have been identified with defects in development of π cells or their daughters. For instance, *egl-13* mutants (also called *cog-2*) fail to properly maintain the π cell fate (Cinar et al., 2003; Hanna-Rose and Han, 1999; Trent et al., 1983). In these mutants, the AC fails to fuse with the utse, presumably as a consequence of the earlier defects. An unfused AC has also been observed in *lin-29* and *lin-11* mutants, which have defects in π cell fate specification and utse differentiation, respectively (Newman et al., 1999, 2000). However, no mutants have been identified that specifically affect fusion of the AC with the utse.

We performed an EMS-based screen for egg-laying defective (Egl) mutants with uterine defects (Cinar et al., 2001, 2003). Here, we describe a mutant from that screen in which the AC fails to fuse with the eight π cell progeny that form the utse. We find that transformation of this mutant with genomic DNA constructs containing the *nsf-1* gene rescues its mutant defects. Our studies regarding the role of *nsf-1* in AC/utse fusion provide a significant connection between the molecules mediating intracellular fusion and the developmental process of intercellular fusion.

Materials and methods

C. elegans handling and strains

Worms used in this study were maintained according to standard procedures (Brenner, 1974). The mutations utilized are listed below. These were isolated by Brenner (1974) unless otherwise indicated.

Linkage group (LG) I: *adr-1(gv6)* (Tonkin et al., 2002), *bli-4(e937)*, *ceh-6 (mg60)* (Burglin and Ruvkun, 2001), *dpy-5(e61)*, *emb-12(g5)*, *emb-19(g22)* (Cassada et al., 1981), *fer-1(hc1)* (Ward and Miwa, 1978), *let-80(s96)*, *let-85 (s142)*, *let-89(s133)* (Rose and Baillie, 1980), *lin-28(n719)* (Ambros and Horvitz, 1984), *rme-8(b1023)* (Zhang et al., 2001), *unc-11(e47)*, *unc-29 (e1072)*, *unc-55(e1170)*, *unc-75(e950)*, *nDf23* (Ferguson and Horvitz, 1985), *unc-13(e51)*, *dxDf1* (Sun and Lambie, 1997).

LG II: *bli-2, dpy-10(e128), eff-1(hy21)* (Mohler et al., 2002), *rab-3(js49, y250, y251)* (Nonet et al., 1997), *snt-1(ad596)* (Avery, 1993), *snt-1(md290, n2665)* (Nonet et al., 1993), *unc-104(e1265)*.

LG III: *dpy-17(e167), rbf-1(js232)* (Staunton et al., 2001), *unc-16(e109), unc-16 (ju146)* (Byrd et al., 2001), *unc-16 (n730)* (Trent et al., 1983), *unc-32 (e189), unc-64(e246), unc-64(md130)* (van Swinderen et al., 1999).

LG IV: *ced-3(n717)* (Ellis and Horvitz, 1986), *dpy-20(e1282), dpy-20 (e1362), unc-26(e205)*.

LG V: *crt-1(bz30)* (Xu et al., 2001), *dpy-11(e224), him-5(e1490), ric-4 (gk312, gk322, gk333), ric-4(md1088)* (Nguyen et al., 1995), *snb-1(md247)* (Miller et al., 1996).

LG X: *lon-2(e678)*.

Mutant isolation and phenotypic analysis

The *ty10* mutant was isolated in an EMS-based screen described previously (Cinar et al., 2001, 2003). The *ty10* mutation used in the studies was outcrossed five to six times.

To measure the percentage of egg-laying defective (Egl) worms, we picked hermaphrodites as L4 larvae onto separate plates. The worms were examined daily for the next 3 days. To obtain the percentage of Pvl (protruding vulva) worms, the same method was employed except that *ty10* worms were examined for 2 days only. To analyze lethality, five hermaphrodites were picked as L4 larvae onto separate plates. The worms were removed the following day and their progeny were examined for the next 3 days. To analyze AC/utse fusion defects, L4 larvae were examined using Nomarski optics. In the case of *eff-1 (hy21)*, L4 larvae were incubated at 25°C for 3 days and their L4 progeny were examined.

Cell fusions were examined in strains expressing AJM-1::GFP. These strains were constructed using strain SU102, containing transgene *jcIs5*, which was made by injection of *ajm-1::GFP, zen-4::GFP* and pRF4.

Invasion of the vulval epithelium by the AC was examined using *dpy-20 (e1362)*; *pkEx246[dpy-20(+)*; *cdh-3::GFP]* (Pettitt et al., 1996).

Nuclear position in the utse was examined using the integrated array *tyIs4* (Cinar et al., 2003), which was made using the *egl-13::GFP* transcriptional gene fusion, pWH17 (Hanna-Rose and Han, 1999).

DNA sequence analysis and constructs

All 13 exons of the *nsf-1* locus were amplified by single worm PCR (Hodgkin, 1999) and both strands of the PCR products were sequenced using 12 primer sets; primer sequences are available upon request.

We used fosmid H15N14 (Sanger Institute) to obtain a 7.7 kb *Bam*HI–*Xho*I fragment of genomic *nsf-1* DNA including 1.6 kb of URS and 0.6 kb of 3′-UTR. This fragment was inserted into pBluescript SK+ (Stratagene) to make the BN1 construct. We also obtained an additional 3.4 kb *Bam*HI–*Bam*HI fragment of *nsf-1* URS. The BN2 construct consists of this fragment inserted into BN1. A 3 kb *Bam*H1–*Sst*II fragment from H15N14 was ligated into the corresponding fragment of BN1 to make the KR1 construct. We then digested the KR1 and BN2 constructs with *Bam*HI. A 3.4 kb *Bam*HI–*Bam*HI fragment from the BN2 construct was ligated into the corresponding fragment of KR1 to create the KR2 construct.

We made the *nsf-1::GFP* construct using *hsp16-CeNSF::GFP* (from M. Nonet), which contains *nsf-1s* genomic DNA behind a heat shock promoter (hsp). This was digested with *Nhe*I, which cleaves at intron 7 of *nsf-1*, and ligated to a *Spe*I–*Nhe*I fragment of BN2 containing the 5 kb URS through intron 7.

We made the *nsf-1l::GFP* construct by introducing a missense mutation (underlined in the primers) into the start codon of *nsf-1s*. We performed an overlapping PCR using Pfx (Invitrogen) and the following two sets of primers: FW1(sense 1,2) – ATGAGTCCAGTCCCGTGTTTAAGC, RV1(nsf-1SKO RV) – TCCGAACACGGAACGCTTTTTCGTTGGACG, FW2(nsf-1SKO FW) – ACGTCCAACGAAAAAGCGTTCCGTGTTCGG, RV2(antisense 6,7) – AACAGTGTCATGGACTGATGAGCTTCC. A *Nhe*I–*Bss*HII fragment from the PCR product was used to replace the corresponding fragment of *nsf-1::GFP*.

To make *nsf-1l::GFP-I*, we digested the *nsf-1::GFP* construct and JBC1 (see below) with *Zra*I and *Nru*I and ligated the fragment from JBC1 containing *nsf-1l* cDNA into the *nsf-1::GFP* vector. To make JBC1, we performed an overlapping

PCR using Pfu (Stratagene), BN2 and yk240h6 cDNA clone (from Y. Kohara) as templates and the following primers: FW1(BN2–5) – AGCCGAATCGTTGAGCG, RV1(BN2–3) – CTTAAACACGGGACTGGACTCATG, FW2 (yk240h6–5) – CATGAGTCCAGTCCCGTGTTTAAG, RV2(yk240h6–3) – GAGAAGATGTTTGATACCGACTCC. A *Nco*I fragment from this PCR product containing *nsf-1* cDNA was ligated into BN2 which had been cut with *Nco*I.

To make *nsf-1l*::*GFP-II*, which has introns 1 and 2 deleted and contains a missense mutation in the start codon of NSF-1S, we digested the *nsf-1l*::*GFP* construct and JBC1 with *Zra*I and *Nru*I and ligated the fragment from JBC1 containing *nsf-1l* cDNA into the *nsf-1l*::*GFP* vector.

To make *nsf-1l*::*GFP-III*, we digested *nsf-1l*::*GFP-I* with *Xho*I and *Bae*I. The fragment containing *nsf-1* was treated with Klenow (NEB). The DNA was self-ligated.

To make the *nsf-1s*::*GFP* construct, we digested BN2 with *Zra*I and ligated this with a *Not*I linker (10-mer, NEB) to introduce a frameshift mutation into the *nsf-1l* coding sequence prior to the start codon of *nsf-1s* in exon 3 of *nsf-1*. A *Mlu*I–*Nhe*I fragment containing the frameshift mutation was used to replace the corresponding fragment of *nsf-1*::*GFP*.

To make *nsf-1s*::*GFP-III*, we performed PCR using the *nsf-1* genomic DNA construct as a template and the following primers: FW(FW1) – ATATATCGATCGTCATGAGTCCAGTCCCG and RV(RVI) – GGCATAATTAGCCAACGTGTGTTC. A *Pvu*I–*Zra*I fragment from the PCR product was ligated into the corresponding fragment (10 kb) of *nsf-1*::*GFP*. Since *nsf-1s*::*GFP-III* contains the distal 1 kb of the 5 kb *nsf-1* URS and exons 1–2, we created *nsf-1s*::*GFP-IV*, which lacks these, as follows. We performed PCR using BN2 as a template and the following primers: FW(*Xho*I-nsf-1sgfpIV FW) – AATCCGGCTCGAGGTTTCGTTTTG and RV(RVI) – GGCATAATTAGCCAACGTGTGTTC. A *Xho*I–*Aat*II fragment from the PCR product was ligated into the corresponding fragment (7.8 kb) of *nsf-1s*::*GFP-III*.

To make the *ACEL*::*nsf-1s*::*GFP* construct, we digested e1417-Δpes-10-(myc)5 tagging vector (from P. Sternberg), which contains AC-specific enhancer of *lin-3* (ACEL) (Hwang and Sternberg, 2004), with *Eco*RV and *Nru*I. The DNA fragment containing ACEL was treated with Klenow and ligated to a *Zra*I–*Not*I fragment of *nsf-1s*::*GFP-I* containing genomic *nsf-1s* DNA tagged with *GFP*.

To make the *ACEL*::*nsf-1l*::*GFP-I* construct, we digested *ACEL*::*nsf-1s*::*GFP* with *Not*I and *Nhe*I. The longer fragment containing ACEL and the second half of *nsf-1s*::*GFP* was purified. Next, we performed a PCR using *nsf-1l*::*GFP-I* as template, FideliTaq (USB) and the following primers: FW(*Not*I–exon1) – GTTTTCAGTGCGGCCGCTTCTCATGAGTCCAG and RV(ty10antisense6,7) – AACAGTGTCATGGACTGATGAGCTTCC. The PCR products were digested with *Not*I and *Nhe*I. This and the *Not*I–*Nhe*I fragment containing ACEL and the second half of *nsf-1s*::*GFP* were ligated together. We also made the *ACEL*::*nsf-1l*::*GFP-II* construct with the same method except that we used *nsf-1l*::*GFP-II* as a PCR template.

To make *egl-13*::*nsf-1s*::*GFP*, we performed PCR using pWH17 (Hanna-Rose and Han, 1999) as a template, FideliTaq, and the following primers: FW (egl-13*Sal*I) – TACGCGTCGACTATAATCGGC and RV(egl-13AatII) – CGTCTAGACGTCATGCTGGAAAAATAC. The PCR products were digested with *Sal*I and *Zra*I. This and the *Xho*I–*Zra*I fragment of *nsf-1*::*GFP* containing *nsf-1s*::*GFP* were ligated together.

To make *rab-3*::*RFP*, we digested pRabGFPrim3′ (from M. Nonet) with *Age*I and *Eag*I and pDsRed2-N1 (Clontech) with *Age*I and *Not*I. The *Age*I–*Not*I fragment containing *RFP* was ligated to the *Age*I–*Eag*I fragment of *rab-3* promoter.

Transgenic rescue

To determine if the constructs containing genomic *nsf-1* DNA rescue *nsf-1 (ty10)* mutant defects, the following constructs were injected into the *nsf-1(ty10)* strain: BN1 (20 ng/μl) with pPD118.20 (20 ng/μl) and pBluescript SK+ (100 ng/μl), BN2 (5.6–20 ng/μl) with pPD118.20 (20–60 ng/μl), KR2 (20 ng/μl) with pPD118.20 (20 ng/μl), *nsf-1*::*GFP* (20 ng/μl) alone (*tyEx5*) or with pBluescript SK+ (100 ng/μl), JBC1 (1–50 ng/μl) with pPD118.20 (17–80 ng/μl) and pBluescript SK+ (0–100 ng/μl), JBC1 (20 ng/μl) with pPD114.108 (20 ng/μl), JBC1 (5 ng/μl) with pWH17 (20 ng/μl), *ACEL*::*nsf-1l*::*GFP-I* (50 ng/μl) with *rab-3*::*RFP* (50 ng/μl) (*tyEx7*) and *ACEL*::*nsf-1l*::*GFP-II* (50 ng/μl) with *rab-3*::*RFP* (50 ng/μl) (*tyEx8*). The following were injected into wild type or *him-5 (e1490)* mutant worms, which were then crossed with *nsf-1(ty10)* to transfer the extra-chromosomal arrays: *nsf-1l*::*GFP-II* (20 ng/μl) with pBluescript SK+ (100 ng/μl), *nsf-1s*::*GFP-III* (20 ng/μl) with pBluscript SK+ (100 ng/μl) (*tyEx11*), *ACEL*::*nsf-1s*::*GFP* (50 ng/μl) with *rab-3*::*RFP* (50 ng/μl) and pBluscript SK+ (100 ng/μl) (*tyEx12*) and *egl-13*::*nsf-1s*::*GFP* (50 ng/μl) with pBluscript SK+ (100 ng/μl) (*tyEx13*).

Analysis of expression patterns

We injected the *nsf-1*::*GFP* (*tyEx5*) construct into N2 worms at 20 ng/μl. Both the *nsf-1l*::*GFP* (*tyEx9*) and *nsf-1s*::*GFP* (*tyEx10*) constructs were injected at 50 ng/μl, while all other constructs were injected at 20 ng/μl with pBluescript SK+ (100 ng/μl, Stratagene).

In addition to the *nsf-1* sequences described in the text, all the above constructs (except *nsf-1s*::*GFP-IV*) also have an hsp and a duplication of exons 3 through 7 of *nsf-1*. However, it is unlikely that the duplication affects the *nsf-1l* expression pattern since *nsf-1l*::*GFP-III*, which is lacking the duplication as well as 300 bp of additional sequence, has the same expression pattern as *nsf-1l*::*GFP-I* (data not shown). Also, neither the duplication nor the distal 1 kb of *nsf-1* URS is likely to affect the expression pattern of *nsf-1s*::*GFP* since *nsf-1s*::*GFP-III*, which contains both the duplication and this region of the URS, results in the same pattern as *nsf-1s*::*GFP-IV*, which lacks them. In addition, we confirmed that the hsp is not functional without heat shock since *hsp16-CeNSF*::*GFP* was not expressed under these conditions. The expression pattern of *nsf-1s*::*GFP-III* was compared to that of *rab-3*::*RFP* to confirm the broad neuronal expression of *nsf-1*.

Western blotting

We prepared whole worm lysates (each with 200 transgenic worms) in standard reducing SDS-PAGE sample buffer by boiling for 8 min. We performed SDS-PAGE using a 5% Tris–HCl gel (Bio-Rad) under reducing conditions. Proteins were transferred onto a PVDF membrane (Bio-Rad). We used the anti-GFP antibody (A.v. peptide antibody; BD biosciences) solution at a concentration of 1 μg/ml and horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody solution (Amersham Biosciences). We developed the membrane using the Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences).

Results

Phenotypic characterization of ty10 mutants

We have been using a genetic screen for Egl mutants to isolate new strains with uterine defects. In particular, we hypothesized that failure to properly develop the utse, or π cells or AC that contribute to it, would cause an inability to lay eggs. This screen has resulted in the isolation of mutants that fail to specify or maintain π cell fates (Cinar et al., 2001, 2003; Newman et al., 2000). In addition, we obtained two mutants with a distinct phenotype specific to the AC. One of these mutants, called *ty10*, is characterized in this study. The other mutant (*ty4*) defines a distinct gene and will be described elsewhere.

The results of our general phenotypic characterization of *ty10* mutants are as follows. We observed 22% embryonic lethality and 23.7% larval lethality ($n = 118$; most worms died at the L1 stage, while two worms were arrested at the L2 stage). We also found that 25% of animals that survived to at least the L4 stage had a protruding vulva at the adult stage (were Pvl; $n = 24$). In addition, of 65 adult hermaphrodites examined, 35.4% failed to lay eggs, with their progeny hatching internally. An additional 55.4% of the hermaphrodites laid eggs less

effectively, leading to egg retention and, in some cases, hatching of embryos within the mother. The final 9.2% of hermaphrodites exhibited egg-laying behavior that was indistinguishable from wild type.

The AC is unfused in ty10 mutants

To assess potential uterine defects, we observed *ty10* mutant hermaphrodites at the L4 stage under Nomarski optics. The thin laminar process of the utse appeared normal (See below for more detail). However, there were striking abnormalities in the appearance of the AC, which is normally fused and indistinguishable from other utse nuclei by the mid-L4 stage. By contrast, in 75.2% of *ty10* mutant animals examined, the AC clearly remained unfused by this stage (n = 101) (Fig. 2). Normally, the AC resides at the apex of the vulva prior to fusion, while the amount of cytoplasm surrounding it appears to decrease. In 31.7% of the *ty10* mutant animals observed, the AC remained dorsal to the vulva but was surrounded by a large mass of cytoplasm and had a "bloated" appearance (Fig. 2B). In 32.7% of animals, the AC was found in a similar position but appeared degenerated (Fig. 2C). In 10.9% of mutant animals observed, the AC appeared to have detached from the adjacent tissue and could be seen floating free in the uterine lumen (Fig. 2D).

To characterize the defect in more detail, we observed eight animals by continuous cell lineage analysis during the fourth larval (L4) stage. Midway during the L4 stage, seven of eight animals had an unfused AC dorsal to the vulva, while one animal was phenotypically wild type. In four of these animals, the AC subsequently detached from the surface of the utse and was observed floating free in the uterine lumen. Two of these animals were observed through L4 lethargus; the AC went through no further changes after detaching from the utse. In the remaining three animals, the AC degenerated and was evident as debris dorsal to the vulva. In the two of these animals observed through L4 lethargus, there were no further changes in the AC.

Another key aspect of uterine–vulval development that requires the AC is invasion of the vulval epithelium by this cell (Sherwood and Sternberg, 2003). To determine whether this process also requires *nsf-1*, we created transgenic *ty10* worms that contain *pkEX246 [dpy-20(+); cdh-3*::*GFP]* (Pettitt et al., 1996). The *cdh-3* gene encodes a member of the cadherin superfamily that is expressed in the AC and is particularly useful in visualizing the cytoplasmic process that can extend from this cell. We found that none of fourteen transgenic *ty10* animals examined had an abnormal contact between the AC and vulval cells (Figs. 2E–I). Specifically, the basement membrane separating the uterus and vulva disappeared at the appropriate time as in *nsf-1(+)* animals. Also, *cdh-3*::*GFP* expression revealed normal contact between the process of the AC and vulval cells.

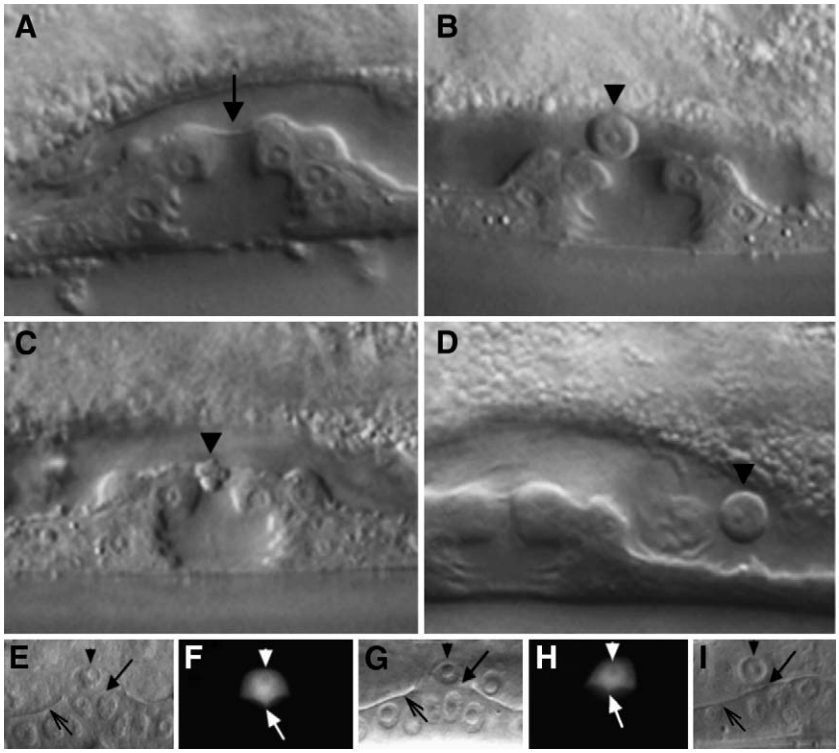

Fig. 2. In *ty10* mutants, the thin laminar process of the utse is observed but the AC remains unfused. (A) Wild-type worm at the L4 stage. Thin laminar process of the utse (arrow) separates the uterine and vulval lumens. The AC has fused with the utse and is not visible as a distinct cell. (B–D) *ty10* mutants at the L4 stage. The arrowheads indicate unfused ACs. (B) The thin laminar process of the utse is evident but the AC is unfused. (C) An unfused AC has degenerated. (D) The AC is unfused and floating free in the uterine lumen. (E–I) *nsf-1(+)* (E, F) and *nsf-1(ty10)* mutant (G, H) worms at the L3 stage. The arrowheads indicate the ACs. The basement membrane (open arrows) has been removed in the vicinity of the AC (arrows in panels E and G). The AC contacts the vulval cells (arrows in panels F and H). The GFP signal corresponds to the expression of the *cdh-3*::*GFP* transcriptional fusion construct from *pkEx246*. (I) For comparison, *fos-1(ar105)* (Seydoux et al., 1993; Sherwood et al., 2005) was examined. The basement membrane (open arrow) remains intact in the vicinity of the AC (arrow).

Degeneration of the AC is not mediated by the caspase-dependent apoptotic or necrotic pathway for cell death in C. elegans

The degeneration of the AC that is observed in some *ty10* mutants suggests that some form of cell death is occurring. The genetic pathways for apoptotic and necrotic cell death have been well characterized in *C. elegans* (reviewed in Hengartner and Horvitz, 1994; Xu et al., 2001). We therefore tested one component of each pathway for possible involvement in AC degeneration using phenotypically null alleles (Ellis and Horvitz, 1986; Park et al., 2001; Shaham et al., 1999; Yuan et al., 1993). To determine if the AC degenerates via the apoptotic cell death pathway, we generated a strain of genotype *ty10; ced-3(n717) unc-26(e205)*. The *ced-3* gene encodes a caspase required for apoptosis (Ellis and Horvitz, 1986). We observed 73% of unfused ACs in the triple mutants; this includes 32.4% worms with a degenerated AC (n = 37; P = 1.000, two-tailed Fisher's exact test). This suggests that AC degeneration in the *ty10* mutant does not require genes of the caspase-dependent apoptotic pathway. To determine if the unfused AC degenerates via the necrotic cell death pathway, we made *ty10; crt-1(bz30)* since *crt-1* (calreticulin) is required for necrosis (Xu et al., 2001). We observed 91.2% of unfused ACs in the double mutants and 44.1% worms with a degenerated AC (n = 34; P = 0.3003, two-tailed Fisher's exact test) implying that AC degeneration is not occurring through the necrotic cell death pathway. We also found that the *ty10* Egl defect was not suppressed by either *ced-3* or *crt-1* (28 out of 29 worms, P = 0.43 and 26 out of 30 worms, P = 0.72, respectively; two-tailed Fisher's exact test).

Other cell fusions appear normal in ty10 mutants

An important question is whether the *ty10* defect affects all nine cells that fuse to form the utse or just fusion of the AC. AJM-1::GFP localizes to adherens junctions (Koppen et al., 2001) and is therefore a useful marker for cell fusion. We observed weak expression of this marker in the utse of *nsf-1(+)* and *nsf-1(ty10)* mutant worms. However, the thin laminar process of the utse was too thin to determine whether cells were fused. We therefore used other approaches to evaluate the overall development of the utse. First, we examined the thin laminar process of the utse in the *ty10* strain under Nomarski optics. We found that the process was similar to wild type in 100 of 101 worms observed. In one of 101 animals, thick tissue was instead observed separating the uterine and vulval lumens. In addition, since the nuclei of the utse probably migrate after both fusions and cell shape changes have occurred (Newman et al., 1996), examination of nuclear position is a reasonable assay for utse development. Since the *egl-13* gene is expressed in the π cell lineage including the nuclei of the utse (Cinar et al., 2003; Hanna-Rose and Han, 1999), we created transgenic *ty10* worms that contain *egl-13::GFP*. These transgenic worms showed a normal number of *egl-13::GFP* positive π cell daughter nuclei (mean = 12.07, n = 41, P = 0.8080, SD = 1.92, two-tailed one sample t test; theoretical number of nuclei in wild type π cell daughters is 12 if the AC were not fused). Also, the location of these nuclei was normal (data not shown), suggesting that the utse had a wild-type shape or cellular composition.

The fact that about a third of *C. elegans* somatic cells fuse (Mohler et al., 2002; Sulston and Horvitz, 1977) raises the question of whether other fusions are defective in *ty10* mutants. *eff-1* mutants are generally defective in cell fusions and are dumpy (Dpy) as a result (Mohler et al., 2002). Since *ty10* mutants are not Dpy, it is likely that fusions in *ty10* mutants are not broadly affected. Nonetheless, we looked directly at cell fusions using AJM-1::GFP. Since most of the fusion events in *C. elegans* occur between epithelial cells, we examined whether epithelial cell fusions for hyp6, hyp7, and seam cells are affected in *ty10* mutants (Fig. 3). An embryo at the comma stage has dorsal epidermis (De), ventral epidermis (Ve), and bilateral seam cells (Se) (Mohler et al., 2002; Podbilewicz and White, 1994). By the two-fold stage, De and Ve cells fuse to become syncytial hyp6 and hyp7 (Mohler et al., 2002; Podbilewicz and White, 1994). A distinct set of epidermal fusions occurs during the L4 stage when the 15 Se cells that form a row on each side of the animal fuse with one another to form one long syncytium per side (Mohler et al., 2002; Podbilewicz and White, 1994). We examined 11 *nsf-1(+)* embryos and 10 *nsf-1(ty10)* mutant embryos at the comma stage and ten *nsf-1(+)* embryos and 12 *nsf-1(ty10)* mutant embryos at the two-fold stage under Nomarski optics or confocal microscopy. The mutant embryos did not show any cell fusion defects in hyp6 or hyp7 (Figs. 3A–D). Next, we examined 14 *nsf-1(+)* worms and 50 *nsf-1(ty10)* mutant worms at the late L4 to young adult stages under Nomarski optics. We observed lateral syncytia rather than discrete cells, suggesting that seam cell fusions also occur normally in *ty10* mutants (Figs. 3E, F). In about 10% of animals, we observed some seam cells missing, with a resulting gap between lateral syncytia. Since alae, which are cuticular structures produced by seam cells, were not observed in the region of gap, the likely cause is a developmental defect in seam cell production and not a cell fusion defect (data not shown). The *C. elegans* epithelium also includes the vulval cells and the Pn.p cells that generate these cells as well as some of the hypodermis. Six of these cells (P3.p–P8.p) are vulval precursor cells (VPCs). By the L3 stage, the progeny of the P(3,4,8).p cells are fused with the hypodermal syncytium while the progeny of the P(5–7).p cells make vulval rings, some of which are syncytial (Sulston and Horvitz, 1977). When we examined 15 *ty10* mutant worms to determine whether the progeny of P3.p, P4.p, and P8.p cells fuse with the hypodermal syncytium, we did not observe cell fusion defects (Fig. 3G). As a control, we also examined cell fusions in 15 *nsf-1(+)* animals (Fig. 3H). We also examined whether the four precursor cells of the outer vulval ring vulA (Sharma-Kishore et al., 1999), which are progeny of the P5.p and P7.p cells, fuse normally in *ty10* mutant worms (n = 20) compared to *nsf-1(+)* worms (n = 14). We did not observe any defects in the fusion of these cells in the *ty10* mutants (Figs. 3I, J). These observations suggest that the *ty10* fusion defect is AC-specific. In addition, M. Nonet (Washington University in St. Louis) performed an aldicarb resistance test to determine if the *ty10* worms were defective in neurotransmitter

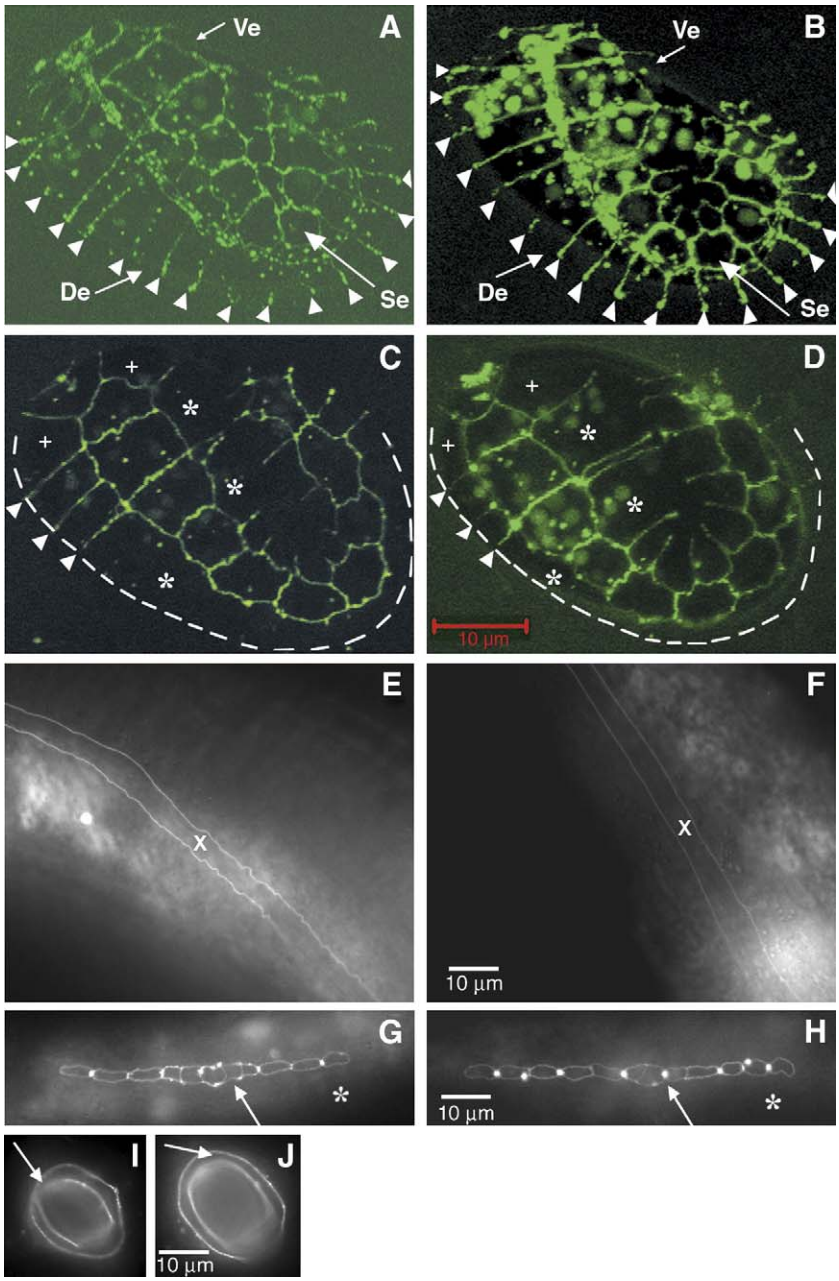

Fig. 3. Epithelial cell fusions are not affected in *ty10* mutants. The signals corresponds to AJM-1::GFP. (A–F) All pictures are lateral views. (A–D) Confocal images. Anterior is left and dorsal is down. (A, C) *nsf-1(+)* embryos. (B, D) *nsf-1(ty10)* mutant embryos. (A, B) Embryos at the comma stage. Cell boundaries are indicated by arrowheads. De: dorsal epidermal cells, Ve: ventral epidermal cells, Se: bilateral seam cells. (C, D) At the two-fold stage, most cell boundaries disappear (dotted line) because De and Ve cells fuse to become syncytial hyp6 (+) and hyp7 (*). The pattern of cell boundaries in *nsf-1(ty10)* embryos is the same as in *nsf-1(+)*. (E, F) GFP fluorescence images of a *nsf-1(+)* worm (E) and a *nsf-1(ty10)* mutant worm (F) observed at the young adult stage. Shown are mid-bodies of the worms. The longitudinal syncytium (X) of 15 Se cells is normal in *ty10*. (G–J) GFP fluorescence images of *nsf-1(+)* (G, I) and *nsf-1(ty10)* mutant (H, J) worms. All pictures are ventral views. (G, H) L3 stage worms showing unfused P5–7.p granddaughters (arrows). The progeny of P3.p, P4.p, and P8.p have fused with their hypodermal syncytium (*) and thus are not apparent as distinct cells. (I, J) L4 stage worms showing the syncytial vulA cell (GFP; indicated with arrows) resulting from the fusion of the four outer ring precursor cells.

release. The test showed no significant difference between wild type and *ty10* mutant worms, suggesting that secretory vesicle transport is also not affected in *ty10* mutants.

AC/utse fusion occurs independently of EFF-1

We examined *eff-1(hy21)* mutant animals to determine whether they show defects in the process of AC/utse fusion. None of 22 mutants were defective in AC fusion. This conclusion has also been confirmed for the *eff-1(np29)* allele (Shemer et al., 2004). We also observed a normal thin laminar process of the utse in the animals that we observed. To further monitor the appearance of the entire utse, we created transgenic *eff-1(hy21)* worms that contain *tyIS4*. These transgenic worms showed a normal number of *egl-13::GFP* positive π cell daughter nuclei (mean = 12.50, n = 18, P = 0.4996, SD = 0.86,

two-tailed paired *t* test) compared to *tyIs4* (mean = 12.83, *n* = 23, SD = 1.15) and a normal location of these nuclei (data not shown). These observations suggest that the utse of *eff-1 (hy21)* has a wild-type shape and cellular composition. Thus, both AC/utse fusion and other aspects of utse development appear to occur independently of *eff-1*.

ty10 corresponds to a missense mutation in the nsf-1 gene

The *ty10* mutation was assigned to chromosome I using two-factor mapping and positioned between *gld-1*(2.30) and *ceh-6* (2.85) based on three-factor mapping, Tc1 mapping, and deficiency mapping data. To identify the gene defined by *ty10*, we injected *ty10* mutant worms with various cosmids in the mapping region (Fig. 4A). We found that only fosmid H15N14 rescued the *ty10* mutant egg-laying defects. We then defined a series of constructs that rescued the *ty10* mutant defects, both in terms of egg laying and AC fusion (Fig. 4B). The only gene completely contained in all constructs was *nsf-1*. Next, we sequenced genomic *nsf-1* DNA from *ty10* mutant worms and found that *nsf-1(ty10)* has a missense mutation (CCA → TCA, Pro647Ser; Seupplementray Fig. S1) in the NSF-D2 ATP binding domain (see Discussion for information on NSF domains) (Fig. 4C), suggesting that the mutant defects are due to the mutation of *nsf-1*, which is the only *C. elegans* gene predicted to encode a member of the NSF family of proteins.

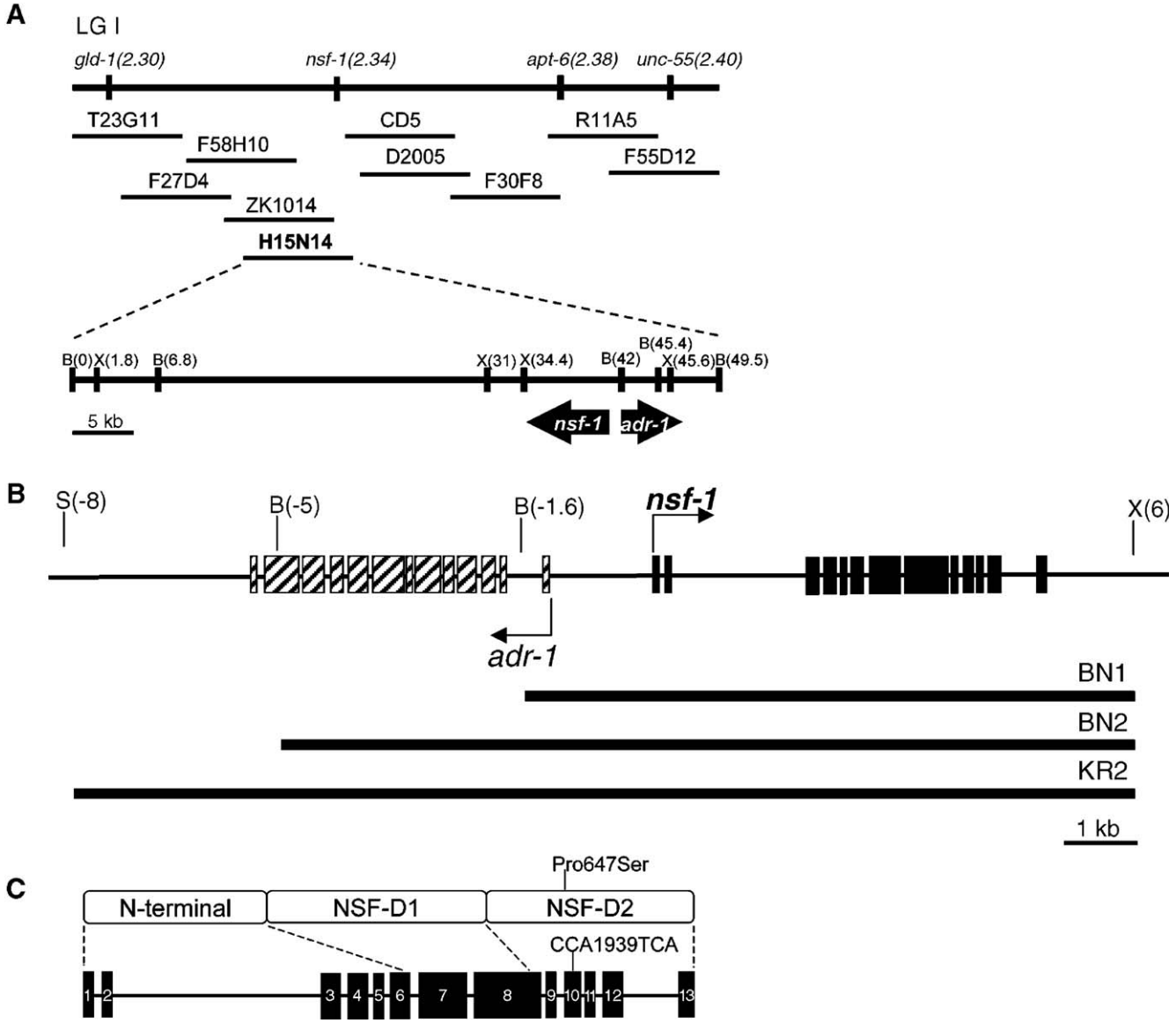

Fig. 4. Fosmid H15N14 and its subclones that contain *nsf-1* genomic DNA rescue the *ty10* mutant defects. (A) Shown are the *nsf-1* mapping region and cosmids which were used to perform transgenic rescue of *ty10* mutant defects. No cosmids other than H15N14 rescued the *ty10* mutant defects. B: *Bam*HI, X: *Xho*I. Numbers in parentheses indicate the distances (kb) from the 5′ end of the fosmid. (B) Scheme of the *nsf-1* genomic DNA constructs generated from the fosmid H15N14 (*nsf-1* is on the forward strand). Constructs BN1, BN2, and KR2 all rescued both the Egl and AC fusion defects of *ty10* mutant worms. Arrows indicate the translational start sites of the designated genes. B: *Bam*HI, S: *Sst*II, X: *Xho*I. Numbers in parentheses indicate the distances (kb) from the translation start site of *nsf-1*. The lines under the names of the rescuing constructs indicate the fragments inserted into pBluescript SK+. (C) Scheme of NSF-1 protein domains aligned with the *nsf-1* exons. *ty10* has a missense mutation in exon 10 (CCA1939TCA; Pro647Ser), which encodes a part of the NSF-D2 domain.

All the above constructs also contain part or all of another gene, *adr-1* (adenosine deaminase acting on RNA, an RNA editing gene) (Fig. 4B), which has been reported to be required for vulval development (Tonkin et al., 2002). Therefore, it was possible that *ty10* was a mutant allele of *adr-1*. However, the following suggest that this is not the case. First, the BN1 construct, which contains *nsf-1* coding sequences plus 1.6 kb of upstream regulatory sequence (URS) of the *nsf-1* gene, rescues *ty10* mutant defects. This construct also contains 75 bp of the first exon and 300 bp of the first intron of *adr-1* genomic DNA and encodes only 25 amino acids of 964 amino acids. Based on the sequence, this construct does not contain any double-stranded RNA-binding motifs (dsRBMs) or catalytic domain, and is therefore likely to be nonfunctional. Second, we performed complementation analysis with *adr-1(gv6)*, a deletion mutant, which is considered to be a null allele since it does not have any dsRBMs or catalytic domain (Tonkin et al., 2002). We found that *nsf-1(ty10)/adr-1(gv6)* heterozygotes appeared wild type, suggesting that *ty10* is not an allele of *adr-1*. By contrast, *nsf-1(ty10)/dxDf1* is lethal (see below). The conclusion that *ty10* is a mutant allele of *nsf-1* is further supported by subsequent experiments demonstrating rescue of the AC fusion defect by expression of only *nsf-1* in the AC (see below).

The nsf-1 gene is required for C. elegans viability

The finding that *nsf-1(ty10)* is a missense mutation raised the possibility that the phenotype we observed might not be the null phenotype. To address this issue, we placed *nsf-1(ty10)* in trans to the deficiency *dxDf1*. Specifically, we performed three crosses between *dpy-5(e61) nsf-1(ty10)* hermaphrodites and *dxDf1/unc-29(e1072)* males. We observed 93 *unc-29(e1072)/dpy-5(e61) nsf-1(ty10)* adult hermaphrodite cross-progeny, all of which were egg-laying competent. By contrast, we observed only five adult hermaphrodite cross-progeny of genotype *dpy-5(e61) nsf-1(ty10)/dxDf1*. Of these, only two had normal egg-laying. We also observed many dead eggs and larvae whose genotype could not be determined. However, the above numbers suggest that many of them contained *nsf-1(ty10)* in trans to the deficiency. We infer that the *nsf-1* null phenotype is more severe than that of *nsf-1(ty10)*, and that *nsf-1* is required for viability.

Consistent with this, elimination of *nsf-1* function through RNA-mediated interference (RNAi) results in lethality (Fraser et al., 2000; Maeda et al., 2001; Poteryaev et al., 2005; our unpublished results). In order to get more functional information on *nsf-1*, we performed complementation analyses using other temperature sensitive or lethal mutants in the region whose molecular identities are not known: *emb-12(g5)*, *emb-19(g22)*, *let-80(s96)*, *let-85(s142)*, and *let-89(s133)*. However, none appeared to be allelic to *nsf-1*.

nsf-1 produces two tissue-specific isoforms through two separate promoters

We examined the localization of *nsf-1* using a gene fusion construct with *GFP* at the C-terminus (Fig. 5A and Table 1) and observed expression in neurons, the pharynx, and the uterine–vulval region (Figs. 5B, C). Analysis of cDNA clones obtained in an EST screen (performed by Y. Kohara) indicated the existence of two classes of *C. elegans nsf-1* transcripts (WormBase, www.wormbase.org), designated *nsf-1l* and *nsf-1s* (Fig. 5A). The longer transcript (*nsf-1l*) has 13 exons while the shorter transcript (*nsf-1s*) is missing the first two exons of *nsf-1l*. In *nsf-1s*, which is in frame with *nsf-1l*, SL1 is *trans*-spliced to exon 3, which contains the *nsf-1s* start codon. Several lines of reasoning suggested that *nsf-1l* and *nsf-1s* might result from transcription through separate promoters rather than from alternative splicing. First, a transcript that normally undergoes *trans*-splicing will instead splice in *cis* if an upstream 5′ splice site is provided (Conrad et al., 1991, 1993). This suggests that the transcription start site of *nsf-1s*, which undergoes *trans*-splicing, might be in intron 2 of the *nsf-1* locus so that the upstream 5′ splice site would not be included in the transcript. Second, intron 2 of *nsf-1l*, which is 1742 bp, significantly exceeds the average intron length (320.8 bp; n = 123,219) (Choi and Newman, in press), and contains many predicted transcription factor binding sites (data not shown). Most of these sites are not found in the 2 kb immediately upstream of exon 1. We, therefore, hypothesized that *nsf-1s* might be transcribed through a separate promoter located in intron 2.

To determine the expression sites of the two *nsf-1* isoforms, we transformed wild type (N2) worms with constructs producing (a) only NSF-1S::GFP due to a frameshift mutation or (b) only NSF-1L::GFP due to a missense mutation in the NSF-1S initiation codon (Figs. 5A, D–I). NSF-1S::GFP is highly expressed in neurons, including those adjacent to the pharynx where a number of neurons gather (Figs. 5D, E). By contrast, NSF-1L::GFP is expressed in the uterine–vulval region, pharynx, and circumpharyngeal nerve ring (Figs. 5F–I). In the uterus, NSF-1L expression is observed in the AC, π cells/daughters, and other uterine cells at the L3 through L4 stages (Figs. 5J, K). In addition to the uterine–vulval expression, we also observed additional gonadal expression, such as the spermathecae and distal tip cells (data not shown). The existence of these distinct expression patterns suggests that *nsf-1s* and *nsf-1l* are regulated by separate promoter regions, which are neuron-specific and pharynx and uterine–vulva-specific, respectively.

To determine whether protein isoforms with the predicted molecular weights (MWs) were expressed in *nsf-1*::*GFP*, *nsf-1l*::*GFP*, and *nsf-1s*::*GFP* transgenic worms, we performed SDS-PAGE and Western blotting using an anti-GFP antibody. The predicted MWs of NSF-1L::GFP and NSF-1S::GFP are 119 kDa and 111 kDa, respectively. Both bands were detected in lysates from *nsf-1*::*GFP* worms, while only the higher MW band was detected in NSF-1L::GFP lysates and only the lower MW band was present in NSF-1S::GFP (Fig. 6). The lower MW band was considerably more intense, consistent with the brighter GFP expression of NSF-1S::GFP.

We next sought to determine the effect of deleting potential regulatory sequences from the wild type genomic *nsf-1* locus. We first inspected transgenic worms containing *nsf-1s*::*GFP-IV*, a construct whose most 5′ sequence is intron 2 (Fig. 7A). We

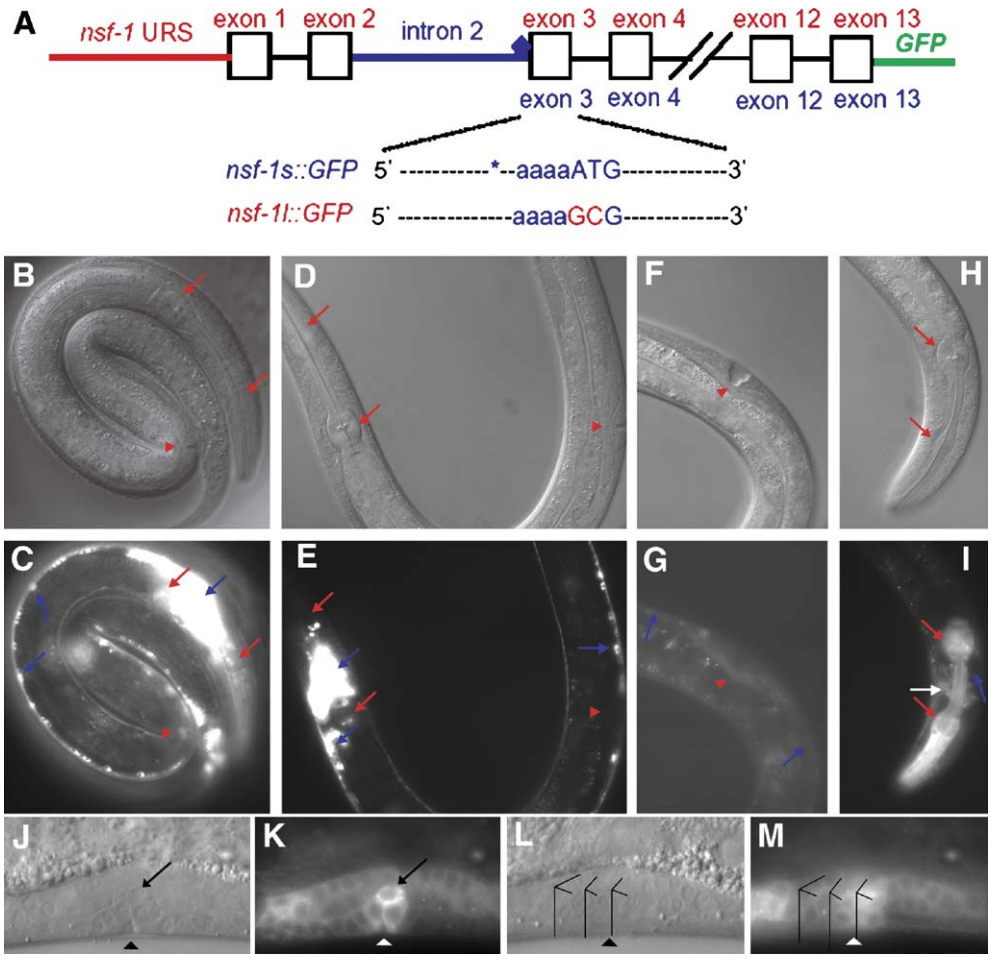

Fig. 5. Expression pattern of *nsf-1* and its isoforms. (A) Gene structure of *nsf-1* and of the *GFP* fusion constructs. Red letters are related to *nsf-1l* and blue to *nsf-1s*. Blue diamond indicates the SL1 acceptor for *nsf-1s* transcript. The *nsf-1*: :*GFP* fusion construct contains genomic *nsf-1* DNA. The *nsf-1s*: :*GFP* construct has a frameshift mutation (blue asterisk) in the *nsf-1l* coding sequence prior to the start codon of *nsf-1s* in exon 3. The *nsf-1l*: :*GFP* construct contains a missense mutation (ATG to GCG; indicated in red) in the start codon of *nsf-1s*. A Kozak consensus sequence (AAAAATG; indicated in blue) is found at the start codon of *nsf-1s*. (B–I) Pairs of Nomarski (upper) and fluorescent (lower) images of transgenic worms. (B, C) NSF-1: :GFP is highly expressed in neurons (blue arrows), the pharynx (red arrow), and the uterine–vulval region (red arrowhead). (D, E) NSF-1S: :GFP is highly expressed in neurons (blue arrows), especially in the vicinity of the pharynx, but has no significant expression in the pharynx itself (red arrow) or in the uterine–vulval region (red arrowhead). (F–I) NSF-1L: :GFP is mainly expressed in the pharynx (red arrow) and the uterine–vulval region (red arrowhead), and is also expressed in the nerve ring (white arrow) but has no significant expression in other neurons (blue arrows). (J–M) Pairs of Nomarski (left) and fluorescent (right) images of *nsf-1l*: :*GFP-III* transgenic worms at the early L4 stage, shortly before fusion of the π cell daughters. NSF-1L: :GFP expression is observed in the AC (arrows) (K) and the π cell daughters (two-pronged lines) (M). Arrowheads point to the vulval center.

observed the same expression pattern as seen in animals containing *nsf-1s*: :*GFP* (Figs. 7B–E). Thus, the 5′-URS and the *nsf-1l*-specific exons/intron are dispensable for the regulation of *nsf-1s* expression. Rather, intron 2 contains an independent promoter that regulates expression of *nsf-1s* and contains the transcription start site of *nsf-1s*. We also examined worms containing *nsf-1l*: :*GFP-I*, which lacks introns 1 and 2 but contains the *nsf-1* URS and *nsf-1l*: :*GFP* (Fig. 7A). We

Table 1
Gene fusion constructs used to study NSF-1 isoform expression

Construct	*nsf-1* URS	Intron 2	Mutation	Expression sites
nsf-1: :*GFP*	Full-length 5 kb	Full-length 1.7 kb	None, both NSF-1L: :GFP and NSF-1S: :GFP are expressed	Pharynx, uterine–vulval region, neurons
nsf-1l: :*GFP*	Full-length 5 kb	Full-length 1.7 kb	Missense mutation in the start codon of *nsf-1s* (Met to Ala), no NSF-1S: :GFP	Pharynx, uterine–vulval region, nerve ring
nsf-1s: :*GFP*	Full-length 5 kb	Full-length 1.7 kb	Frameshift mutation, no NSF-1L: :GFP	Neurons
nsf-1l: :*GFP-I*	Full-length 5 kb	None	None	Pharynx, uterine–vulval region, nerve ring
nsf-1s: :*GFP-IV*	None	Full-length 1.7 kb	None	Neurons

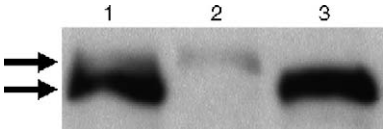

Fig. 6. Western blot analysis of transgenic strains using anti-GFP antibody. Shown are lysates from the *nsf-1*: :*GFP* (lane 1), *nsf-1l*: :*GFP* (lane 2), and *nsf-1s*: :*GFP* (lane 3) transgenic strains. Both bands migrated between the 81 kDa and 135 kDa MW bands from the marker lane, consistent with their predicted MW.

observed robust GFP expression in the pharynx, the uterine–vulval region, and the nerve ring, but no significant expression in other neural tissues (Figs. 7F–I). Thus, intron 2 is not necessary for expression of *nsf-1l*. The above results suggest that expression of the two NSF-1 isoforms is regulated by two separate promoters.

AC/utse fusion requires nsf-1 expression in the AC, not the utse

We next sought to determine whether NSF-1S or NSF-1L was the mediator of AC/utse fusion. To test whether NSF-1S rescues the AC fusion defect of *nsf-1(ty10)* worms, we injected N2 worms with *nsf-1s*: :*GFP-III* and then crossed the transformed lines with *nsf-1(ty10)* worms to transfer the extrachromosomal array to the mutant worms. We found that this construct did not rescue the AC fusion defects (37 out of 48 had unfused ACs compared to 76 unfused ACs out of 101 in *nsf-1(ty10)*; $P = 0.841$, two-tailed Fisher's exact test). We next sought to test whether NSF-1L rescues the AC fusion defect. To do this, we tried to generate *ty10* animals expressing NSF-1L: :GFP. However, we were unable to obtain fertile transgenic *ty10* animals. This may result from the titering of transcription factors at the intronic promoter of *nsf-1s*, which may be more deleterious in combination with the *ty10* mutation.

To circumvent the above problems and to determine whether NSF expression in the AC was sufficient for cell fusion, we constructed transgenic animals in which NSF-1L fused to GFP was expressed only in the AC. We did this using ACEL (AC-specific enhancer of *lin-3*) (Hwang and Sternberg, 2004), which has been shown to be responsible for the AC expression of *lin-3*. We made two separate constructs differing in the start codon of NSF-1S, *ACEL*: :*nsf-1l*: :*GFP-I* (no mutation) and *ACEL*: :*nsf-1l*: :*GFP-II* (a missense mutation in the start codon of NSF-1S). 23 out of 29 *ty10* worms with *ACEL*: :*nsf-1l*: :*GFP-I* had fused ACs ($P = 1.52 \times 10^{-7}$, two-tailed Fisher's exact test) and 13 out of 14 *ty10* worms with

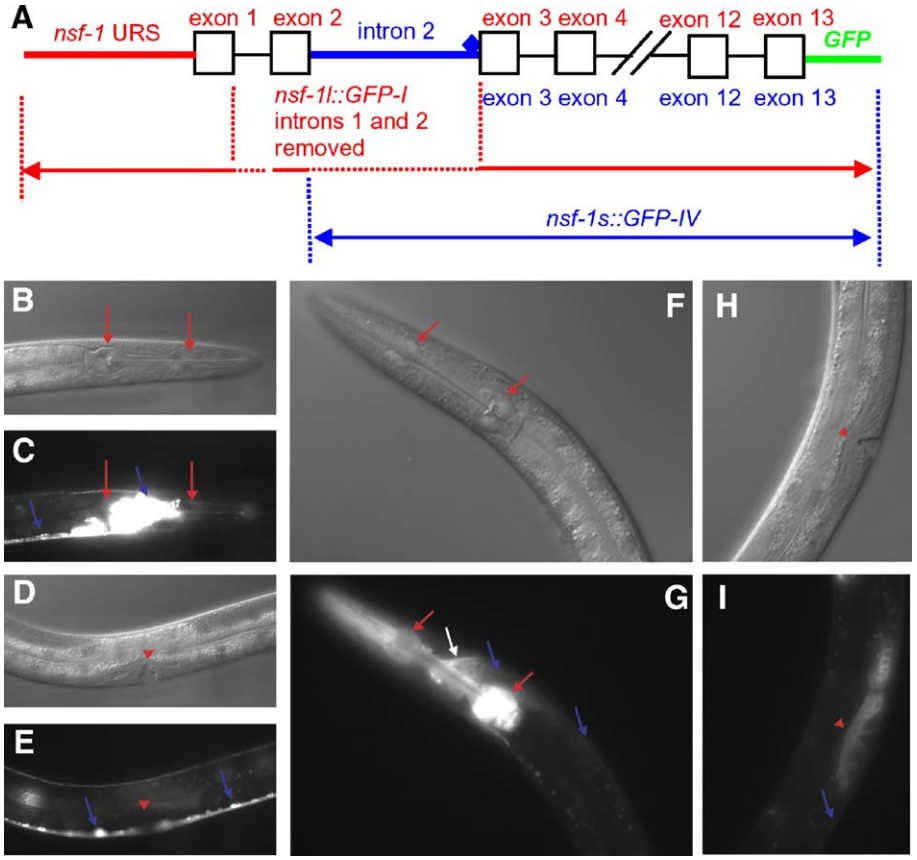

Fig. 7. *nsf-1* expression is regulated by two separate promoters. (A) *GFP* fusion constructs. *nsf-1l*: :*GFP-I* contains neither introns 1 nor 2 (dotted lines in red arrow), but is otherwise identical to *nsf-1*: :*GFP*. *nsf-1s*: :*GFP-IV* contains intron 2 and all genomic *nsf-1* sequences C-terminal to this. (B–I) Pairs of Nomarski (upper) and fluorescent (lower) images of transgenic worms. (B–E) *nsf-1s*: :*GFP-IV* shows the same expression pattern as the *nsf-1s*: :*GFP* construct (Fig. 5), with high expression in neurons (blue arrows) and no significant pharyngeal (red arrow) or uterine–vulval (red arrowhead) expression. (F–I) *nsf-1l*: :*GFP-I* is expressed in the pharynx (red arrow), the uterine–vulval region (red arrowhead), and in the nerve ring (white arrow) but not in other neurons (blue arrows).

ACEL::*nsf-1l*::*GFP-II* had fused ACs ($P = 1.2 \times 10^{-6}$, two-tailed Fisher's exact test). Thus, NSF-1L can rescue the AC fusion defect. In addition, these results suggest that *nsf-1l* functions cell-autonomously in the AC.

As discussed above, transgenes containing only *nsf-1s* do not rescue the *ty10* AC fusion defect. Since NSF-1S is not normally expressed in the AC, these experiments do not address whether the NSF-1S protein can substitute for NSF-1L, which contains an additional 66 N-terminal amino acids. We therefore constructed *ACEL*::*nsf-1s*::*GFP* and created transgenic *ty10* worms containing this construct. The ectopic expression of NSF-1S in the AC also rescued the AC fusion defect (30 out of 33 worms had fused ACs; $P = 1.03 \times 10^{-11}$, two-tailed Fisher's exact test), suggesting that NSF-1S is functionally substitutive for NSF-1L.

Although AC-specific expression of either NSF-1L or NSF-1S can rescue the cell fusion defect, in neither case was the Egl defect rescued (19 out of 23 worms, $P = 0.669$ and 23 out of 24 worms, $P = 0.28$, respectively; two-tailed Fisher's exact test). Thus, the unfused AC appears not to be the sole obstacle to egg laying in *nsf-1(ty10)* mutant worms. Since the egg-laying system requires neurons, muscles, and the proper structure of the uterus and vulva, it seems that *nsf-1(ty10)* worms also have defect(s) in additional tissues that we have not detected.

We wondered whether NSF-1 expression in the utse might also rescue the AC/utse fusion defect. We therefore generated an *egl-13*::*nsf-1s*::*GFP* construct since the *egl-13* promoter has been shown to direct expression in the utse (Cinar et al., 2003; Hanna-Rose and Han, 1999). We found that the AC fusion defect was not rescued in transgenic *nsf-1(ty10)* worms (2 out of 24 worms had fused ACs; $P = 0.1001$, two-tailed Fisher's exact test). Thus, it appears that the AC, and not the π cell daughters with which it fuses, requires *nsf-1* for fusion of the AC with the utse.

Discussion

The ty10 mutant has specific defects in fusion of the AC

The *C. elegans* utse is formed by the fusion of nine uterine cells: eight π cell daughters and the AC. Prior to this, the AC induces both the vulva and the π cells whose daughters connect to it, thus organizing the uterine–vulval connection. Data from electron micrographic reconstructions suggest that the π cell daughters fuse first with each other and then with the AC (Newman et al., 1996). However, since the molecular basis for these fusions had not been elucidated, it was unclear whether or not both types were controlled by the same genes. We have now isolated a mutant with specific defects in fusion of the AC with the utse. The existence of such a mutant implies that this fusion is genetically distinct from other utse cell fusions. This establishes fusion of the AC with the utse as a paradigm for studying cell fusion at single cell resolution.

Subsequent to its failure to fuse, the AC either degenerates or detaches from the uterine lumen. We found that degeneration of the AC does not require either the apoptotic or necrotic pathway for cell death in *C. elegans*.

The nsf-1 gene is required for fusion of the AC with the utse

NSF has been known to function in intracellular secretory vesicle fusion by disassembling SNARE complexes. In addition, the acrosome reaction, a form of exocytosis required for sperm–egg fusion, also requires NSF (Michaut et al., 2000; Tomes et al., 2005). In this study, we have described a role for NSF in intercellular membrane fusion. The *ty10* mutation is the first *C. elegans NSF* allele identified. As expected from the functions of NSFs in other organisms, *C. elegans* NSF is also essential for viability. Surprisingly, however, we have found that while the AC fails to fuse with the utse in *nsf-1(ty10)* mutants, other cell–cell fusions appear normal. Based on rescue and expression data, *nsf-1* is required cell-autonomously in the AC for its fusion with the utse, and NSF-1L is responsible for this event.

NSF-1 is an AAA family member (ATPases Associated with diverse cellular Activities) and contains three functional domains: N-terminal, D1, and D2 (Fig. 4C; see Seupplementray Fig. S1 for further information) (Matveeva et al., 1997; reviewed in May et al., 2001; Nagiec et al., 1995; Steel and Morgan, 1998; Sumida et al., 1994; Whiteheart et al., 1994). Both the D1 and D2 domains contain consensus sequences for ATP binding and hydrolysis; however, the major roles of D1 and D2 are ATPase and ATP binding, respectively (Whiteheart et al., 1994). In addition, the D2 domain is required for hexamerization of NSF; however, only complete deletion of this domain inhibits hexamerization (Block et al., 1988; Nagiec et al., 1995; Whiteheart et al., 1994). NSF molecules with mutations in the D2 domain have significant levels of vesicle fusion activity (Sumida et al., 1994; Whiteheart et al., 1994; reviewed in Whiteheart and Matveeva, 2004). Consistent with this, we found that the *nsf-1(ty10)* mutation, which corresponds to a missense mutation in the D2 domain, does not cause defects in neurotransmitter release. Nonetheless, while the effect of the *ty10* mutation appears to be sufficiently subtle so that most NSF-1 functions are unperturbed, the consequences for the AC are dramatic.

Tissue-specific expression of two NSF-1 isoforms

In this study, we have demonstrated the tissue-specific expression of two NSF-1 isoforms. The isoforms differ by 66 N-terminal amino acids, which are present in NSF-1L, but not in NSF-1S. It is the NSF-1L isoform that is expressed in the AC. The first 66 amino acids of NSF-1L do not share homology with NSFs from other organisms (Supplementary Fig. S1). Since, in general, the N-terminal domain of NSF is involved in substrate binding, it is possible that the additional amino acids of NSF-1L might function in the recognition of additional substrates. The data showing that NSF-1S is substitutive for NSF-1L in the AC argue against this hypothesis. Nevertheless, since NSF-1S was overexpressed from an extrachromosomal array in this experiment, it is unclear whether NSF-1S recognizes the appropriate substrate as efficiently as does NSF-1L. Interestingly, we have also found that the two isoforms are expressed under the control of two separate promoters.

How does NSF-1 work during AC/utse fusion?

One way that NSF could function in AC/utse fusion is through a mechanism involving the secretory vesicle pathway. However, when we examined other mutants that have defects in secretory vesicle transport and/or fusion, we did not observe an AC fusion defect. These include *rab-3(js49, y250, y251)* (Ras GTPase), *rbf-1 (js232)* (rabphilin-3A), *ric-4(gk312, gk322, gk333, md1088)* (SNAP-25), *snb-1(md247)* (synaptobrevin), *snt-1(ad596, md290, n2665)* (synaptotagmin), *unc-11(e47)* (clathrin-adaptor protein), *unc-16(e109, ju146, n730)* (vesicle trafficking protein), *unc-64(e246, md130)* (syntaxin), and *unc-104(e1265)* (kinesin-like protein, axonal transporter of synaptic vesicles). We also examined *rme-8(b1023)*, which has endocytosis defects, and performed RNAi of α-SNAP, which functions with NSF in worms and other organisms (Li et al., 2004; reviewed in Whiteheart and Matveeva, 2004). However, we did not observe defects in AC fusion. As described in the Introduction, the AC transmits signals to other cells through secreted or membrane-bound proteins during the AC/VU decision as well as later in uterine and vulval development. *nsf-1(ty10)* mutants show no defects in these cell fate decisions, suggesting that the secretory pathway is functional in the AC. In addition, we found that AC invasion of the vulval epithelium (Sherwood et al., 2005) was normal in *ty10* mutants. This implies that the proteins that are involved in the removal of basement membrane are normally transported to the plasma membrane or secreted. Moreover, the fact that the overall development of *nsf-1(ty10)* mutant worms and the level of aldicarb resistance appear similar to wild type suggests that many intracellular and extracellular events that depend on the secretory pathway are occurring normally. Taken together, the above observations suggest that NSF-1L might not function through the secretory vesicle transport pathway in the AC. Nonetheless, we cannot rule out the possibility that combinations of secretory pathway proteins might function redundantly in AC fusion.

A second possibility is that NSF-1 functions outside the cells to resolve unknown surface SNARE complexes that mediate fusion between the AC and the utse. Consistent with this, Rothman and colleagues showed that the expression of SNAREs on the outside of cells could cause cell fusion (Hu et al., 2003). By using SignalP (a signal peptide prediction program) (Nielsen et al., 1997), we found that NSF did not have a signal peptide. Nonetheless, it is possible that NSF-1 might be secreted through nonclassical secretion pathways, which do not require signal sequences (reviewed in Muesch et al., 1990; Rubartelli et al., 1990). However, the finding that expression of NSF-1 in the AC and not in the utse rescues the AC fusion defect of *nsf-1(ty10)* suggests that NSF-1 is not likely to be secreted and function outside of cells (see Fig. 1A for the relevant topology).

An attractive possibility is that NSF-1 functions directly on non-SNARE cell surface protein(s), possibly AC fusion factor(s), to alter their conformation or interaction with other proteins. As discussed earlier, NSF has been previously shown to function on non-SNARE targets. It is likely that *C. elegans* has fusion factor (s) that have not yet been identified since both fertilization and AC fusion occur independently of EFF-1. These findings suggest that the AC may require its own unique fusogenic protein(s) and/or fusion mechanism with which NSF-1, specifically NSF-1L, functions. One way to identify such proteins is to screen for additional mutants with defects similar to those of *ty10*. In fact, we have obtained one such mutant which, by genetic criteria, defines a distinct gene. Cloning and characterization of this gene may provide insight into the nature of the NSF substrate.

Recent findings suggest that vacuolar-H^+ATPases (V-ATPases) may, like NSF, function both in intracellular membrane fusion and in cell–cell fusion (Bayer et al., 2003; Hiesinger et al., 2005; Kontani et al., 2005; Peters et al., 2001; Pujol et al., 2001). Of particular relevance to this study is the observation that the *C. elegans* V-ATPase functions during cell–cell fusion as a negative regulator of EFF-1 (Kontani et al., 2005). It would be very interesting to determine whether there is any functional interaction between NSF and V-ATPase during cell–cell fusion.

Overall, this study establishes AC/utse cell fusion as a paradigm for studying how cells fuse, and demonstrates that NSF is a key factor in this process. In the future, identification of additional components necessary for AC fusion, as well as their mode of interaction with NSF, will provide important insight into the critical process of cell fusion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.471.

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